AD_____

Award Number: DAMD17-03-1-0077

TITLE: Development and Pre-clinical evaluation of a novel Prostate-Restricted Replication Competent Adenovirus-Ad-IU-1

PRINCIPAL INVESTIGATOR: Thomas A. Gardner, M.D.

CONTRACTING ORGANIZATION: Indiana University
Indianapolis IN 46202-5167

REPORT DATE: May 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| **1. REPORT DATE** *(DD-MM-YYYY)* | **2. REPORT TYPE** | **3. DATES COVERED** *(From - To)* |
|---|---|---|
| 01-05-2005 | Annual | 01 May 04 - 30 Apr 05 |

**4. TITLE AND SUBTITLE**
Development and Pre-clinical evaluation of a novel Prostate-Restricted Replication Competent Adenovirus-Ad-IU-1

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
DAMD17-03-1-0077

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**
Thomas A. Gardner, M.D.

E-Mail: thagarnd@iupui.edi

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Indiana University
Indianapolis IN 46202-5167

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT** Recently we generated a prostate specific chemic promoter, called PSES, by combining the active prostate specific enhancers from prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) genes which are prominently expressed in androgen independent (AI) prostate cancers. The goal of this research is to develop a novel therapeutic agent, Ad-IU-1, using PSES to control the replication of adenovirus and the expression of a therapeutic gene, herpes simplex thymidine kinase (TK). AD-IU-1 replicates as efficient as a wild type adenovirus in PSA/PSMA positive cells, but not in PSA/PSMA negative cells. Prodrug GCV augmented Ad-IU-1's killing activity against PSA/PSMA positive cells, but not PSA/PSMA negative cells in vitro. Ad-IU-1 was more effective in inhibit the growth of androgen-independent CWR22rv tumors. Due to recent improvement in our adenoviral vector construction which allows us to insert a bigger transgene into the viral genome, we further investigated a fusing suicide gene, FCYttk, by combining two suicide genes, a yeast cytosine deaminase, FCY, and improved TK, ttk. FCYttk had a better killing activity than TK against prostate cancer cells. We have constructed and investigated a FCYttk-armed prostate restricted replicative adenovirus for future clinical investigation.

**15. SUBJECT TERMS**
TRANSCRIPTIONAL REGULATION OF ADENOVIRAL REPLICATION, GENE THERAPY, PSES CHIMERIC PROMOTER, TISSUE RESTRICTED REPLICATION COMPETENT ADENOVIRUS, MICROPET IMAGING

| **16. SECURITY CLASSIFICATION OF:** | | | **17. LIMITATION OF ABSTRACT** | **18. NUMBER OF PAGES** | **19a. NAME OF RESPONSIBLE PERSON** |
|---|---|---|---|---|---|
| **a. REPORT** | **b. ABSTRACT** | **c. THIS PAGE** | | | USAMRMC |
| U | U | U | UU | 38 | **19b. TELEPHONE NUMBER** *(include area code)* |

## TABLE OF CONTENTS

# INTRODUCTION

Metastatic human prostate cancer (PC) is commonly treated by hormone, radiation, and/or chemotherapy. Inevitably, these patients will eventually relapse and develop androgen-independent (AI) disease with osseous metastasis. Since no effective therapy is presently available for the treatment of PC metastasis, we are developing a novel gene therapy modality for hormonal refractory prostate cancer based on a prostate-specific chemic promoter, PSES, generated in collaboration with Dr. Chinghai Kao, a co-investigator of this project. In this study, we proposed to generate a herpes simplex virus thymidine kinase (TK) armed prostate restricted replicative adenovirus (PRRA) to treat AI prostate cancers. Specific Aim 1 intended to simplify and combine the most important enhancer elements from PSA (prostate specific antigen) and PSMA (prostate specific membrane antigen) enhnacers/promoters to generate a strong and simple prostate-specific chimeric enhancer, sPSES. Specific Aim 2 would test whether sPSES retained prostate specific activity in an adenoviral vector. Specific Aim 3 would test whether sPSES could control adenoviral replication by controlling adenovirus E1a and E1b expression, and investigate how replication competent adenovirus eradicated prostate cancers by micro positron emission tomography (microPET) imaging.

# BODY

**Task 1.** To generate a strong and simple prostate-specific enhancer. (Months 1-6):

a. Generate deletion consrtuct (Months 1-4). We have successfully deleted L2 and L5 in AREc3 (Figure 1), and replaced the 90 bp proximal region


**Figure 1.** Sequences of the enhancer core of the PSA gene, AREc3, located the 4.3 kb upstream of PSA promoter. Sequence analysis on AREc3 revealed 6 putative GATA transcriptional factor binding sites besides reported 3 androgen response elements.

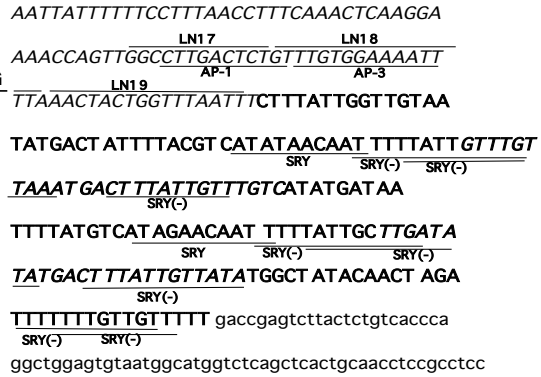



**Figure 2.** Sequences of PSME located in the third intron in the PSMA gene (*FOLH*1). PSME is characterized by the repeat sequence (marked by underline and bold) and an Alu sequence (marked by lower case). Several potential transcription factor binding sites, such as AP-1, AP-3, and SRY/SOX, are indicated.

(marked by italic in Figure 2) of PSME with simple AP-3 binding site. These manipulations reduced the size of PSES from 513 bp to 407 bp. The simplified version of PSES is called m6.

b. Test the tissue-specificity of new deletion constructs (Months 3-6). The tissue specific activity of m6 has been tested in several cell lines. Figure 3 demonstrated that m6 retained a strong prostate specificity, active only in PSA/PSMA positive prostate cancer LNCaP and C4-2 cells.

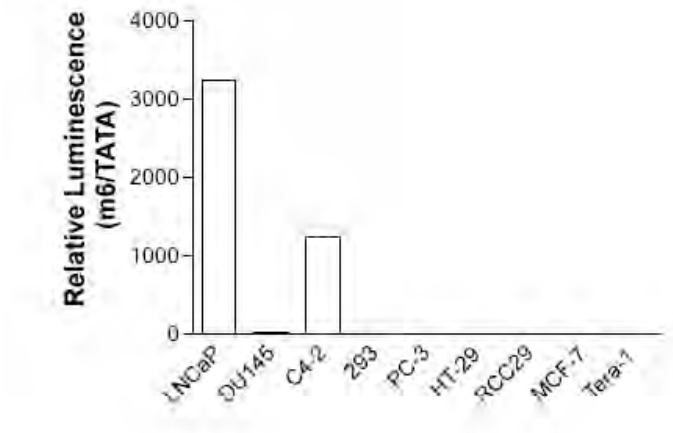



**Figure 3.** pGL3/m6/TATA (0.5 μg) was transfected into various cell lines (2 x $10^5$ cells for each). After 2 days, cells were harvested, lysed with passive lysis buffer (Promega) and analyzed for luciferase activities. This experiment was conducted in the absence of androgen. The luciferase activity was determined by being divided by the basal activity represented by transfection of pGL3/TATA. pGL3/m6/TATA is avtive only in PSA/PSMA postivie LNCaP and C4-2 cells.

Task 2. To test tissue-specificity of the modified PSES chimeric enhancer, m6, in an adenoviral vector (Months 7-24):

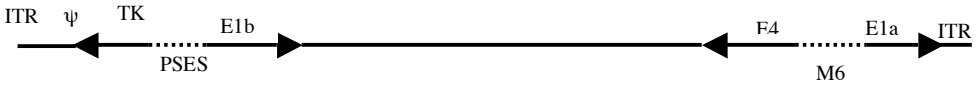
a. Construct Ad-m6-Luc (Months 7-12). Ad-m6-Luc has been generated.

b. Test the tissue-specificity of Ad-m6-Luc in tissue culture cells (Months 13-18). Ad-m6-Luc exhibited the same prostate specificity in all cell lines tested. However, this study was not continued due to a technical difficulty in making Ad-IU-1 initially (see discussion below).

c. Test the tissue-specificity of Ad-m6-Luc in vivo (Months 19-22). This study was not continued due to a technical difficulty in making Ad-IU-1 initially (see discussion below).

Task 3. Investigate the capability of m6 to drive adenovirus replication in a prostate cancer-specific manner (Months 13-36):

a. Construct Ad-IU-1 (Months 7-16). The first attempt to make Ad-IU-1 according to the original plan has failed due to a size limitation of adenoviral vector. We have changed our construction strategy and made Ad-IU-1 as illustrated below.




b. In vitro test tissue-specific expression of TK, E1a and E1b proteins in Ad-IU-1 infected cells (Months 17-20). As expected, AD-IU-1 mediated E1a and TK expression only in PSA/PSMA positive cells (see Figure 2 in the attached manuscript). Due to unavailability of anti-E1b anti-body and E1b is under the control of the same promoter as E1a, we did not investigate whether E1b expression from Ad-IU-1 was specific to PSA/PSMA positive cells.

c. In vitro test the tissue specific replication of Ad-IU-1 (Months 19-22). Ad-IU-1 replicated as efficient as AdE4PSESE1a, a PSES controlled replicative adenovirus, in PSA/PSMA positive cells, and its replication efficiency was dramatically attenuated in PSA/PSMA negative cells (see Table 1).

d. In vitro test the therapeutic efficacy of Ad-IU-1 (Months 20-23). Ad-IU-1 had less toxicity than AdE4PSESE1a in treating PSA/PSMA positive cell in the absence of prodrug GCV; on the other hand, Ad-IU-1 was 10 to 100 fold more potent than AdE4PSESE1a in killing PSA/PSMA positive cell in the absence of the prodrug ganciclovir, GCV. Ad-IU-1 had minimal toxicity toward PSA/PSMA negative cells DU145 in the presence or absence of GCV (see Figure 3 & 4 in the attached manuscript).

e. In vivo test the therapeutic efficacy of Ad-IU-1 (Months 22-36). CWR22rv subcutaneous tumors in the castrated nude mice were used to test the therapeutic efficacy of Ad-IU-1. The therapeutic efficacy of Ad-IU-1 was significantly enhanced by GCV. Without GCV, Ad-IU-1 has a similar therapeutic effect as AdE4PSESE1a on CWR22rv tumors (see Figure 5 and 6 in the attached manuscript).

f. MicroPet image C4-2 tumors (Months 9-20). It has been finished and published (see Reportable Outcomes section). In general, microPET imaging of tumor is less sensitive than PSA assay. The reviewers of this proposal also pointed out this problem.

g. MicroPET image the therapeutic effect of Ad-IU-1 (Months 21-36). We have made 8-[$^{18}$F]-fluoropenciclovir for TK microPET imaging study (see Reportable Outcomes section). However, due to the unexpected delay of this project, this study is still ongoing.

Additional results: It has been reported that cytosine deaminase (CD)- thymdine kinase (TK) fusion fusion gene (CD/TK) exert a much better therapeutic effect than CD and TK themselves. CD/TK-based gene therapy has been studied extensively in clinic to treat prostate cancer by Svend O. Freytag (1-6). We constructed a fusion gene, FCYttk, between a yeast cytosine deaminase (FCY) and a modified thymidine kinase (ttk), an N-terminal deleted form of sr39TK. FCYttk demonstrated better killing activity than bacterial CD when supplemented with the prodrug 5-FC, and demonstrated similar killing activity to

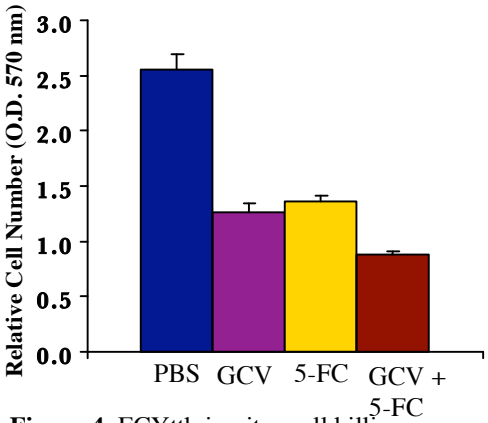



**Figure 4.** FCYttk in vitro cell killing assay. CWR22rv cells were seeded in a 24-well ($1x10^5$ cells/well) plate and transfected with pAd1020PSESFCYttk. The cells were treated with GCV (5 ug/mL), 5-FC (25 uM) 24 hours after transfection. Medium was changed every 2 days. At 5day, cell number was analyzed using crystal violet staining. GCV at 5 $\mu$g/ml and 5-FC at 25 uM concentration are no-toxic to CRW22rv (unpublished data).

sr39TK when supplemented with the prodrug, GCV (unpublished data). We transfected pcDNA3.1-FCYttk expression vector into CWR22rv cells and treated the cells with PBS, GCV, 5-FC or GCV+5-FC. FCYttk/GCV/5-FC treatment exerted much stronger killing activity than FCYttk/GCV or FCYttk/5-FC treatment alone (Figure 4). We are on the process of constructing a FCYttk-armed prostate-restricted replicative adenovirus.

## h. KEY RESEARCH ACCOMPLISHMENTS

1. Successfully shorten PSES as planned and demonstrate the prostate specific activity of the shortened form of PSES, m6.

2. Successfully construct Ad-m6-Luc and Ad-IU-1.

3. Have tested [$^{11}$C]-choline and [$^{18}$F]-FDG microPET imaging in prostate cancer nude mice model.

4. Successfully synthesize 9-(4-[18F]fluoro-3-hydroxymethylbutyl)guanine ([18F]FHBG) for microPET imaging Ad-IU-1 activity.

5. Ad-IU-1 demonstrated a prostate specific replication and killing activity.

6. The tumor killing activity of Ad-IU-1 is significantly enhanced by GCV as hypothesized originally.

## REPORTABLE OUTCOMES

Some of the results of this study have been published.

1. Q-H. Zheng, **T.A. Gardner,** S.P. Raikwar, C. Kao, K.L. Stone, T.D. Martinez, B.H. Mock, X. Fei, J-Q. Wang, G.D. Hutchins: [11C]Choline as a PET biomarker for assessment of prostate cancer tumor models. *Bioorg. Med. Chem.* **2004**, *12*, 2887-2893.

2. J-Q. Wang, Q-H. Zheng, X. Fei, X. Liu, **T.A. Gardner,** C. Kao, S.P. Raikwar, B.E. Glick-Wilson, M.L. Sullivan, B.H. Mock, G.D. Hutchins: An improved total synthesis of PET HSV-tk gene expression imaging agent 9-[(3-[18F]fluoro-1-hydroxy-2-propoxy)methyl]guanine ([18F]FHPG). *Syn. Commun.* **2004**, *34(5)*, 917-932.

3. Q-H. Zheng, J-Q. Wang, X. Liu, X. Fei, B.H. Mock, B.E. Glick-Wilson, M.L. Sullivan, S.P. Raikwar, **T.A. Gardner,** C. Kao, G.D. Hutchins: An improved total synthesis of PET HSV-tk gene reporter probe 9-(4-[18F]fluoro-3-hydroxymethylbutyl)guanine ([18F]FHBG). *Syn. Commun.*, **2004**, *34(4)*, 689-704.

## CONCLUSIONS

We have successfully shortened PSES as planned and demonstrated the prostate specific activity of the shortened form of PSES, m6. We made a prostate restricted replicative adenovirus-armed with TK, Ad-IU-1, as proposed. Ad-IU-1 replicated efficiently in PSA/PSMA positive prostate cancer cells. Its replication efficiency was significantly attenuated in PSA/PSMA negative cells. Both in vitro and in vivo tumor-killing activities of Ad-IU-1 are significantly enhanced by the

prodrug GCV as our original hypothesis. We are on the process of conducting a toxicology studies to prepare Ad-IU-1 for clinical investigation. At the same time, we are improving Ad-IU-1 by replacing it TK gene with a more powerful suicide gene, FCYttk.

## REFERENCES


1. Rogulski, K. R., Kim, J. H., Kim, S. H., and Freytag, S. O. Glioma cells transduced with an Escherichia coli CD/SHV-1 TK fusion gene exhibit enhanced metabolic sucide and radiosensitivity. Human Gene Therapy, *8:* 73-85, 1997.
2. Freytag, S. O., Rogulski, K. R., Paielli, D. L., Gilbert, J. D., and Kim, J. H. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy [see comments]. Hum Gene Ther, *9:* 1323-1333, 1998.
3. Rogulski, K. R., Wing, M. S., Paielli, D. L., Gilbert, J. D., Kim, J. H., and Freytag, S. O. Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. Hum Gene Ther, *11:* 67-76., 2000.
4. Barton, K. N., Paielli, D., Zhang, Y., Koul, S., Brown, S. L., Lu, M., Seely, J., Kim, J. H., and Freytag, S. O. Second-generation replication-competent oncolytic adenovirus armed with improved suicide genes and ADP gene demonstrates greater efficacy without increased toxicity. Mol Ther, *13:* 347-356, 2006.
5. Freytag, S. O., Stricker, H., Pegg, J., Paielli, D., Pradhan, D. G., Peabody, J., DePeralta-Venturina, M., Xia, X., Brown, S., Lu, M., and Kim, J. H. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. Cancer Res, *63:* 7497-7506, 2003.
6. Freytag, S. O., Khil, M., Stricker, H., Peabody, J., Menon, M., DePeralta-Venturina, M., Nafziger, D., Pegg, J., Paielli, D., Brown, S., Barton, K., Lu, M., Aguilar-Cordova, E., and Kim, J. H. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. Cancer Res, *62:* 4968-4976, 2002.

# Tumor-specific suicide gene therapy for prostate cancer using PSES promoter-driven herpes simplex virus thymidine kinase and ganciclovir

Miwon Ahn,[1,4] Xiong Li,[1,4] Sang-Jin Lee,[1,4] Juan Antonio Jiménez,[1,2,4] Yan-Ping Zhang,[1,4] Kyung-Hee Bae,[1,4] Yousef Mohammadi[3], Chinghai Kao,[1,2,4] and Thomas A. Gardner[1,2,4]

Departments of [1]Urology, [2]Microbiology and Immunology, [3]Biology and [4]Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana.

Corresponding Author:

Thomas A. Gardner, MD
Indiana University School of Medicine
Department of Urology
535 Barnhill Drive
Suite 420
Indianapolis, IN 46202
Phone: 317-278-3434; Fax: 317-274-0174; E-mail: thagardn@iupui.edu

Running Title: Tumor specific suicide gene therapy for prostate cancer

Key words: Suicide gene therapy, PSA, PSMA, prostate-specific promoter, PSES, HSV-TK, GCV

**Abstract**

Enzyme prodrug suicide gene therapy has been hindered by gene delivery and transduction efficiency. In order to further explore the potential of this approach, we have developed a prostate-restricted replicative adenovirus (PRRA) armed with the herpes simplex virus thymidine kinase (HSV-TK). This suicide gene (*HSV-TK*) and prodrug (ganciclovir (GCV)) combination has been extensively explored in both preclinical and clinical studies. In our previous Ad-OC-TK/ACV phase I clinical trial, we demonstrated both safety and proof of principle using a tissue-specific promoter based TK/prodrug therapy in treating metastatic prostate cancers. In this study, we aimed to inhibit the growth of androgen-independent (AI), PSA/PSMA-positive prostate cancer cells by AdIU1, an HSV-TK-armed prostate-restricted replicative adenovirus (PRRA). *In vitro*, the growth of an androgen-independent PSA/PSMA-expressing prostate cancer cell line, CWR22rv, was significantly inhibited by treatment with AdIU1 plus GCV (10 μg/ml), as compared to AdIU1 treatment alone. On the other hand, AdE4PSESE1a (a PRRA lacking HSV-TK) with and without GCV (10 μg/ml) treatment demonstrated similar *in vitro* cytotoxicity as AdIU1 alone. *In vitro* cytotoxicity was observed following treatment with AdIU1 plus GCV only in PSA/PSMA-positive CWR22rv and C4-2 cells, but not the PSA/PSMA-negative cell line, DU-145. *In vivo* assessment of AdIU1 plus GCV treatment revealed a therapeutic effect against CWR22rv tumors in nude mice, which was not seen following treatment with AdIU1 alone, AdE4PSESE1a or AdE4PSESE1a plus GCV. In summary, we developed a novel therapeutic strategy for the treatment of AI prostate cancer.

## Introduction

Prostate cancer is still remains the leading cancer diagnosis in men. The incidence of prostate cancer is age-dependent and has steadily increased over the last several decades [1]. Localized prostate cancer can be managed effectively with surgery or radiation, while advanced and metastatic disease eventually progresses to an androgen-independent (AI) state with limited treatment options. The aging population of men with an increasing prostate cancer incidence combined with an absence of successful therapies for advanced disease require the development of novel therapies.

Tumor-specific suicide gene therapy using a tissue-specific promoter is a rational treatment strategy for prostate cancer [2,3]. Herpes simplex virus *thymidine kinase* (*HSV-TK*)-based suicide gene therapy has been used to target prostate cancer for over a decade [4-6]. The prodrug ganciclovir (GCV) is phosphorylated by HSV-TK to its monophosphate form, which is rapidly converted to di- and triphosphate forms by cellular kinases, the latter of which is toxic to cells. The GCV- triphosphate is incorporated into DNA during cell division, causing single-strand DNA breaks and inhibition of DNA polymerase [7]. Prior preclinical studies have demonstrated that HSV-TK/prodrug-based suicide gene therapy inhibited the growth of mouse and human prostate cancer cells *in vivo* [8]. Clinical studies using the HSV-TK/GCV system for the treatment of prostate cancer have also shown the effectiveness of this suicide gene therapy strategy [9]. However, previous HSV-TK/GCV studies suffered from inefficient gene transduction rates *in vivo*.

Tumor-specific oncolytic adenoviruses have been effective and safe treatment options for patients with metastatic disease. We have demonstrated that both the prostate-specific antigen (PSA) and osteocalcin (OC) promoters could transcriptionally regulate the HSV-TK gene in a prostate-specific manner. This tissue-specific HSV-TK production combined with prodrug administration inhibited the growth of Al-PSA-producing cells in vitro, in animal models of

human prostate cancer and in patients with prostate cancer enrolled in a Phase I clinical trial of the OC promoter-based HSV-TK gene therapy [10, 11]. Others have demonstrated similar *in vitro* and *in vivo* efficacy as well as safe administration to men with locally advanced and metastatic prostate cancer[12, 13]. More recently, Freytag *et. al.* have demonstrated the safety and efficacy of a conditionally replicating, non-tissue-specific adenovirus containing the suicide genes *TK* and *CD* when combined with external beam radio therapy [14-16]. Although Ad5-CD/TKrep/prodrugs/radiation therapy demonstrated a promising result locally recurrent prostate cancer, the expression of the CD/TK fusion gene under the control of strong universal CMV promoter lack of tissue specificity severely impairs the safety of this virus.

We recently develop a prostate-specific chimeric enhancer, *PSES*, by combining enhancers from *PSA* and *PSMA* genes. Prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) are prostate-specific biomarkers that are expressed by the majority of prostate tumors and non-cancerous epithelium. The main prostate-specific enhancer activity of the PSA enhancer core was located in a 189 bp region called AREc3, and the main prostate-specific enhancer activity of the PSMA enhancer core was located in a 331 bp region called PSME(del2). The combination of these two regulatory element, AREc3 and PSME(del2), called PSES, showed high activity specifically in PSA/PSMA-positive prostate cancer cells, regardless of androgen status [17]. This *PSES* promoter has been used to control the replication of a PRRA, which demonstrated prostate-specific replication and therapeutic efficacy both *in vitro* and *in vivo* [18]. In this study, we developed a novel *HSV-TK*-armed replicative adenovirus, AdIU1, using the *PSES* promoter to drive the expression of adenoviral *E1a*, *E1b* and *E4* genes in addition to *HSV-TK*. AdIU1 demonstrated its selective cytotoxicity toward androgen-independent (AI) PSA/PSMA-expressing prostate cancer both *in vitro* and *in vivo*.

## Materials and Methods

### Cells and Cell Culture

HER 911E4 cells are derived from adenoviral *E1* (bp 79 to 5,789)-immortalized HER 911 (human embryonic retinoblastoma) cells that stably express adenoviral E4 proteins under control of the *tetR* promoter. HER 911E4 cells were cultured in DMEM, supplemented with 10 % FBS, 1 % penicillin/streptomycin, 0.1 mg/mL hygromycin B (Calbiochem, San Diego, CA) and 2 μg/mL doxycycline (Sigma, St. Louis, MO). AI, androgen receptor (AR) - and PSA/PSMA-positive prostate cancer cell lines C4-2 and CWR22rv, and AI, AR- and PSA-negative cell lines DU-145 and PC3 were cultured in RPMI 1645 supplemented with 10 % FBS and 1 % penicillin/streptomycin. The cells were maintained at 37°C in a 5 % $CO_2$ incubator.

### Construction of the prostate-restricted replicative andenovirus (PRRA), AdIU1.

The construction of the backbone for AdIU1, AdE4PSESE4, was described previously[18]. To construct AdIU1, the *CMV-EGFP* expression cassette in AdE4PSESE1a was replaced by a *PSES-HSV-TK* expression cassette. *HSV-TK* and *E1b* in the left arm and *E4* and *E1a* in the right arm were placed under the transcriptional control of *PSES*. Figure 1 illustrates the structure of the each virus used in this study. The adenoviral genome was released from the cloning vector by digestion with Pac I restriction enzyme and transfected into HER 911E4 cells using Lipofectamine 2000 transfection reagent (Invitrogen, CA, USA). The plate was incubated at 37°C under 5 % $CO_2$ for 7 to 10 days after transfection, until a cytopathic effect was observed. AdIU1 was further amplified in HER 911E4 cells. The recombinant adenoviruses were purified by CsCl gradient centrifugation. All gradient-purified viral stocks were dialyzed in dialysis buffer (1 mM $MgCl_2$, 10 mM Triz HCl (pH 7.5) and 10 % glycerol) for 24 hrs at 4°C, changing the buffer three times. Aliquots of purified virus were stored at -70°C.

**Viral replication assay**

CWR22rv, C4-2, PC-3 and DU-145 cells were seeded in 6-well plates (1 x $10^6$ cells per well) 1 day prior to viral infection and subsequently infected with AdIU1 or AdE4PSESE1a (1000 virus particles (vp)/cell). The media were changed 24 hrs after infection, and the viral supernatants were harvested 3 days after infection. The cells were examined under light microscopy daily for up to 5 days. Then, the titers of the harvested viral supernatants were determined by titer assay. HER 911E4 cells were seeded in 96-well plates (5 x $10^3$ cells per well) 1 day prior to infection. The cells were infected with serial volume dilutions of the harvested supernatants, ranging from 1 to $10^{-11}$ μL per well, with each row of 8-wells receiving the same dose of virus. The media were changed on day 4, and the cells were examined under the microscope on day 7. The dose of the produced viruses was represented as an $LD_{50}$ value, the dilution factor that caused a cytopathic effect in at least 4 wells of cells in a single row on a 96-well plate by day 7.

**Western blot analyses for AdIU1**

For Ad5E1a and HSV-TK protein detection, 1x $10^6$ cells plated in 60 mm dishes were infected with 1000 v.p/cell of AdIU1. Cells were harvested and lysed in 100 μL of cell lysis buffer (50 mM Tris-HCl (pH 7.4), 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 μg/mL Aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM $Na_3VO_4$, and 1 mM NaF) 24 and 48 hrs post viral infection. Lysates were centrifuged at 14000 rpm for 20 min. and the supernatants were collected. Total protein was estimated by dye binding assay (Bio-Rad, Hercules, CA). Protein (25 μg) was loaded onto 10 % SDS-PAGE gel. The protein were transferred to polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Hercules, CA) and the

membrane was probed with antibodies reactive to Ad5E1a protein (BD Bioscience, ) or TK polyclonal serum ( provided by M. Black, Department of Pharmaceutical Sciences, Washington State University, Pullman, WA). And visualized by an enhanced chemiluminescence kit (Amersham Life Science, Piscataway, Nj).

**Dose-dependent *in vitro* cell killing assay**

CWR22rv and DU145 cells were seeded onto 24-well plates at a density of 1.5 x $10^5$ or 1 x $10^4$ cells/well respectively. After 24 hr, the cells were infected with 0.1-1000 vp per cell of AdIU1 or AdE4PSESE1a. 24 hr after infection, the media were removed and replaced by fresh media with or without GCV (10 μg/ml). Media with or without GCV were changed every 2 days. Viable cells were analyzed by crystal violet assay 7 days post-infection.

**Time-dependent *in vitro* killing assay**

CWR22rv and DU-145 cells were plated in 24-well plates. Cells were divided into 4 treatment groups, no treatment, AdIU1 (100 vp/cell), GCV, and AdIU1 (100 vp/cell) plus GCV. The media were changed 24 hrs after infection, and GCV (10 μg/ml) was added 24 hrs after the media change. Cell viability was analyzed at day 1, 3, 5 and 7 by crystal violet assay.

***In vivo* evaluation of AdIU1 therapy**

All animal methods and procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (IACUC). CWR22rv xenografts were established by injecting 2 x$10^6$ cells s.c. in the flanks of 6 week-old male, athymic nude mice. The injected mice were castrated 3 days after cells injection. Mice with similar tumor sizes (3 – 5 mm) were divided into four groups receving, AdE4PSESE1a (negative control PRRA),

AdE4PSESE1a plus GCV, AdIU1, or AdIU1 plus GCV treatment. 2 x $10^9$ v.p. of either AdE4PSESE1a or AdIU1 in 100 μL 1xPBS were injected intratumorally. 5 days after virus injection, GCV (40 mg/kg) was administered i.p. twice-daily for 10 days. Tumor sizes were measured every 5 days, and the following formula was applied to calculate tumor volume, length x width$^2$ x 0.5236. Mice were sacrificed and tumors harvested for histological examination 30 days after injection.

**Histology and Immunohistochemistry**

Tumors were harvested, immediately fixed in formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (H&E), according to the standard protocol. For immunohistochemistry, tumor sections were deparaffinized, rehydrated and heated in a microwave oven for 20 min in activity antigen-retrieval solution (10 mM citric buffer, pH 6.0). Endogenous peroxidase was inactivated with 3% hydrogen peroxide solution. The slides were rinsed with distilled water, washed twice with PBS for 3 min and incubated with Superblock (Scytek Laboratories, Burlingame, CA, USA) in a humidified chamber for 1 hr at room temperature. After rinsing with PBS, the slides were incubated with avidin (Vector Laboratories, Inc., Burlingame, CA, USA) for 15 min, washed with PBS and blocked with biotin in a humidified chamber for 15 min at room temperature. A monoclonal mouse antibody to adenovirus type 5 (Abcam, Cambridge, MA, USA) and a mouse monoclonal antibody Ki67 antigen (Novovastra Laboratories Ltd., UK) were applied. These slides were incubated with primary antibodies overnight in humidified chambers at 4 °C. After PBS rinse, a biotinylated secondary antibody was applied to the slides and incubated for 1hr. After washing with PBS, slides were incubated with avidin-peroxidase complex (ABC) reagent (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min, washed once with PBS, stained with freshly prepared

diaminobenzidin (DAB) solution for 15 min and counterstained with hematoxylin.

***In situ* Terminal Deoxynucleotide Transferase-Mediated Nick End Labeling Assay**.

The *in situ* apoptosis detection kit was purchased from Roche Diagnostics (Indianapolis, IN). Tumor tissue sections were deparafinized using a sequential xylene protocol and rehydrated through gradients of ethanol and distilled water. Slides were treated with 10 nmol/L Tris solution containing 1 μg/ml proteinase K for 15 min. All slides were rinsed with PBS and incubated with 100 μL terminal deoxynucleotidyl transfererase-mediated nick end labeling (TUNEL) reaction mixture (or 100 μL control labeling solution for negative control) in a humid chamber at 37$^{o}$C for 30 min. The slides were washed 3 times with PBS and incubated with 100 μL TUNEL POD solution in a humid chamber at 37$^{o}$C for 30 min. After washing with PBS, the slides were stained with freshly prepared DAB solution for 10 min, rinsed with PBS, and counterstained with hematoxylin.

## Results

### Construction of a TK-armed PRRA.

AdIU1 was constructed by replacing the *CMV-GFP* expression cassette in AdE4PSESE1a [18] with a *PSES-HSV-TK* expression cassette to extend the therapeutic potential of the PSES-based PRRA (Fig 1). HER 911E4 cells were transfected with recombinant adenoviral plasmid linearized by Pac I restriction enzyme digestion, and AdIU1 was purified from the harvest viral supernatant. To assess the prostate-specificity and viral replication efficiency of AdIU1, we performed an *in vitro* viral replication assay. PSA/PSMA-positive and – negative cells were infected with AdIU1. Viral plaques were observed only in the PSA/PSMA-positive cells (Table 1), demonstrating the fact that AdIU1 replication is tightly controlled by PSES and restricted to PSA/PSMA-positive cells.

### Western blotting analysis of adenovirus E1a and herpes simplex virus thymidine kinase proteins expression.

AI, PSA/PSMA-positive CWR22rv and C4-2, as well as AI, PSA/PSMA-negative DU-145 and PC 3 were infected with 1000 v.p / cell. 24hrs and 48hrs post AdIU 1 infection, cell lysate was collected and western blot was performed using monoclonal antibodies against Ad5E1a or polyclonal HSV1-TK antiserum. Ad5E1a and HSV1-TK proteins expression were detected in AdIU1 infected, PSA/PSMA positive CWR 22rv and C 4-2 cells. On the other hand, PSA/PSMA negative DU 145 and PC3 cells, their expression was low o undetectable in AdIU1 infected (Fig 2). This result indicated that PSES promoter retained it prostate specificity to mediated E1a and HSV-TK expression in AI, PSA/PSMA positive cells.

### Selective cell killing activity of AdIU1 plus GCV against AI, PSA/PSMA-positive human

**prostate cancer cells *in vitro*.**

We performed a prodrug sensitivity assay *in vitro*. Each cell line, CWR22rv, C4-2 and DU-145 were seeded in triplicate in 24-well plates at a density of $2 \times 10^4$ cells/well and were incubated with increasing concentrations of GCV (0 to 100 μg/ml). Cell viability was determined after 5 days using crystal violet assay, and a corresponding $IC_{50}$ dose was determined for each cell line (data not shown). We determined the optimal GCV treatment dose to be 10 μg/ml.

To evaluate the selective cytotoxicity of AdIU1 and AdE4PSESE1a viruses, we infected each cell line with wide dose ranges (0.1-1000 v.p./cell) of virus, and then treated infected cells with or without GCV (10 μg/ml) (Fig 3). The growth of AI, PSA/PSMA-positive human prostate cancer cell, CWR22rv was significantly inhibited by 0.1 v.p. of AdIU1 in the presence of GCV. AdIU1 without GCV had similar killing activity as AdE4PSESE1a (negative control) either in the presence or absence of GCV. The growth of AI, AR- and PSA/PSMA-negative cell line, DU 145 was unaffected by either virus with or without GCV.

**AdIU1/GCV showed cytotoxicity in AI, PSA/PSMA-positive cancer cells**

CWR22rv (PSA/PSMA-positive) and DU-145 (PSA/PSMA-negative) cells were seeded in-24 well plates. The 24 wells were divided into 4 groups, no treatment, AdIU1, GCV alone, and AdIU1 plus GCV treatment. The GCV alone group demonstrated limited cytotoxicity. This confirmed that 10 μg/mL GCV treatment was not toxic to either prostate cancer cell lines. The CWR22rv cell line demonstrated cell growth inhibition at day 7 after AdIU1 exposure, which was significantly enhanced when GCV was administered following AdIU1 infection. The DU-145 cell line demonstrated limited cytotoxicity in all four groups investigated (Fig 4).

**In vivo growth inhibition of CWR22rv xenograft by AdIU1/GCV**

Human prostate CWR22rv xenograft tumors were induced by subcutaneous injection of CWR22rv cells into athymic nude mice. The mice were castrated 3 days after CWR22rv inoculation to test whether AdIU1 or AdE4PSESE1a was able to eliminate AI tumors in a castrated host. After tumor formation, the mice were randomized into 4 groups (AdIU1, AdE4PSESE1a, AdIU1 plus GCV and AdE4PSESE1a plus GCV). The mice were injected intratumorally with AdIU1 or AdE4PSESE1a as a control. Day 0 was the time of virus injection. On day 5, groups receiving GCV treatments were injected with GCV (40 mg/kg) 2 times a day for 10 days. Tumor volumes were measured at the times indicated in Figure 4. AdIU1/GCV effectively caused growth delay of CWR22rv xenografts. Light microscopic observation of H&E-stained tumors in mice injected with AdIU1/GCV showed substantial treatment effect (Fig 6). We also observed a large number of fibrosis following combined treatment. To compare proliferation between the GCV treatment group and non-treated group, we performed immunohistochemistry using the proliferation antigen, Ki67. We observed no significant difference in proliferation antigen expression between both groups. Also, we observed that all necrotic tumors stained positive for apoptosis by TUNEL assay. Anti-adenovirus type 5 E1a immunohistochemical staining revealed that extensive viral infection existed throughout the AdIU1, AdE4PSESE1a, and AdE4PSESE1a plus GCV treatment group tumors; however, adenovirus staining was absent in the AdIU1 plus GCV treatment group.

**Discussion**

Replication-defective recombinant adenoviruses have been widely studied *in vitro* and *in vivo* as a vector to deliver cancer therapeutic genes. Adenoviral based cancer gene therapy still maintains unrealized potential. The ability to infect and transduce a variety of mammalian cells, including prostate cells [11] in a cell cycle replication-independent manner without genotoxicity. However, there are several limitations in the use of these vectors for cancer gene therapy.

The current investigations build on the ability of the adenovirus to infect prostate cancer cells and provide both expanded infection and longer exogenous gene expression with a prostate restricted replication-competent oncolytic virus, AdIU1. AdIU1 can replicate and kill infected cells by viral lysis, leading to *in vivo* amplification of input viral dose, spreading to adjacent cancer cells after lysis of initially infected cells. Additionally, AdIU1-infected cells produce HSV-TK to enhance killing with prodrug administration and allow for imaging of the viral process [19]. Several studies have demonstrated the importance of tissue-specific vectors, revealing systemic toxicity with the administration of high doses of nonspecific vectors. Through the use of prostate-specific promoters and enhancers, the expression of a therapeutic gene or adenoviral replication can be limited to cells that contain the appropriate activators and transcription factors. Currently, *kallikrein 2*, *PSA*, *rat probasin*, and *osteocalcin (OC)* are each under extensive investigation as regulators of prostate restricted replication adenovirus [20-22]. In our previous investigations, prostate-specific enhance sequence (PSES), was developed by locating the minimal sequence, AREc3 and PSME(del2) in AREc and PSME, respectively and placing AREc3 upstream from PSME (del2)[17]. PSES showed high activity specifically in PSA/PSMA-positive and AI prostate cancer cells[18].

Gene therapy with *HSV-TK* as a suicide gene has been performed in a variety of tumor models *in vitro* as well as in *vivo*. We already showed that both *PSA* and *OC* promoters can

trascriptionally regulate *HSV-TK* gene-based therapy to inhibit the growth of AI PSA-producing cells. In this study, we investigated the gene-directed enzyme/prodrug therapeutic effect of AdIU1 (a novel PRRA expressing *PSES* promoter-driven *HSV-TK* suicide gene).

The *in vitro* tissue-specific cytotoxicity of AdIU1/GCV (10 μg/ml) in CWR22rv, C4-2 and DU-145 cells were assessed. Whereas the growth of CWR22rv and C4-2 cell lines were significantly inhibited by a small number of AdIU1 virus particles and GCV, the growth of the DU 145 could only be inhibited by a much greater exposure to AdIU1/GCV, this was expected, as DU-145 cells are PSA/PSMA-negative prostate cancer cells.

Collectively these results demonstrate that AdIU1/GCV has selective cytotoxicity in AI, PSA/PSMA-expressing cells with a good therapeutic window. As expected, intratumoral injection of AdIU1 and treatment with GCV effectively induced growth delay of CWR22rv tumors in nude mice. H&E staining revealed a large number of fibroblasts infiltrate within the virus plus prodrug treatment groups. Furthermore, AdIU1/GCV-treated tumors were significantly inhibited in growth. Prostate cancer varies widely in its biological behavior, ranging from slowly growing tumors to aggressive metastasising cancers. so that, it evaluate any change in the proliferative capacity of tumor cells that could more accurately charaterize the biological change in a prostate cancer during and after treatment such as gene therapy. Ki-67 nuclear immunohistochemical staining is related to the biological aggressiveness of tumors and the clinical prognosis of patients with various cancers[23]. To compare proliferation between GCV-treated tumors and non-treated tumors, we performed immunohistochemistry using the proliferation antigen, Ki67 [2, 23, 24]. We observed no different expression proliferation each groups. We observed heavy necrosis and apoptosis induction in combined treated groups.

In conclusion, we have established a prostate-restricted replication-competent adenovirus using *PSES* promoter to drive both oncolysis and *HSV-TK* expression only in AI PSA/PSMA-

positive prostate cancer cells. Gene therapy as monotherapy against prostate cancer currently remains in its infancy. Although preventive strategies are being entertained, the ultimate clinical use of gene therapy for improving cancer treatment would most likely be in combination with surgery, radiation or chemotherapy in prostate cancer. So, our next step is combined approaches to prostate cancer including enzyme prodrug suicide gene therapy such as, AdIU 1 with GCV and radiation therapy or AdIL-12 cytokine therapy for improving conventional prostate cancer treatment represent the most promising immediate clinical application for prostate gene therapy.

**Figure Legend**

**Figure 1. Schematic illustration of AdIU1**. AdIU1 was constructed by placing adenoviral E1a and E4 genes under the control of PSES to direct adenovirus replication, and HSV-TK gene, a pro-drug enzyme gene, under the control of another copy of PSES enhancer to restrict specific expression to maximize cell-killing activity through a bystander effect under the catalysis of gancivlovir (GCV).

**Figure 2. Expression of Ad5 E1a and HSV1-TK proteins by AdIU1 was evaluated in different cell lines.** CWR22rv, C4-2, PC 3 and DU 145 were infected with 1000 v.p/cell. 24 and 48 hrs post viral infection, cell lysate were collected and western blot was performed. A large amount of E1a proteins ranging in size from approximately 35-46 kDa were detected in CWR 22rv and C 4-2 cells. Also CWR 22rv and C 4-2 was expressed TK.

**Figure 3. In Vitro Killing assay.** 1.5 X $10^5$ CWR22rv and DU145 cells were seeded in 24-well plates and infected by serial dilutions of AdIU1 from 0.1v.p/cell to 1000 v.p/cell, with replicative-deficient adenovirus AdE1aPSESE4 as controls. Crystal violet staining was performed to detect attached cells. Then 1% SDS was added to lyses the cells and for $OD_{590}$ reading. Cell survival rate curves were drawn to evaluate the killing activity of AdIU-1.

**Figure 4. In vitro cytotoxicity assay.** Growth of cells, infected with 100 virus particle/cell of AdIU1 virus, was significantly inhibited by addition of Ganciclovir (GCV) (10 μg/mL), especially in CWR22rv(a). The other hand DU 145 (b) cells had no effect.

**Figure 5. Effect of antitumor of AdIU1 in vivo.** CWR22rv prostate tumor xenografts was established S.C. in athymic nude mice. Tumors were treated with AdE4PSESE1a (n=7), AdE4PSESE1a+GCV (n=8), AdIU1 (n=8) or AdIU1+GCV (n=8) by intratumoral injection at day 0 and after 5 days, injected GCV (80 mg/kg of body weight/day) 2times a day for 10 days. And tumor volumes were measured every 5 days.

**Figure 6**. **Tumors were removed for histological examination after the mice were killed at 30 days.** (a) AdIU1 plus GCV, (b) AdIU1, (C) AdE4PSESE1a plus GCV and (d) AdE4PSESE1a treatment group (H&E, X 2). These slide showed a lot of fibroblast GCV treatment groups, also AdIU1 with GCV group showed small number of necrosis spot and tumor volume is very small. All necrosis spot show the apoptotic signal by the TUNEL Assay. AdIU1 with GCV group, E1a expression is rare. Proliferation antigen, Ki67 immunohistochemistry, we observed no different expression proliferation each groups.

**References**


1. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin.* Mar-Apr 2006;56(2):106-130.
2. Cheon J, Kim HK, Moon DG, Yoon DK, Cho JH, Koh SK. Adenovirus-mediated suicide-gene therapy using the herpes simplex virus thymidine kinase gene in cell and animal models of human prostate cancer: changes in tumour cell proliferative activity. *BJU Int.* Apr 2000;85(6):759-766.
3. van der Poel HG, McCadden J, Verhaegh GW, et al. A novel method for the determination of basal gene expression of tissue-specific promoters: an analysis of prostate-specific promoters. *Cancer Gene Ther.* Dec 2001;8(12):927-935.
4. Xing Y, Lu G, Xiao Y, et al. [Bystander effect mediated by herpes simplex virus-thymidine kinase/ganciclovir approach on prostatic cancer cells and its regulation]. *Zhonghua Yi Xue Za Zhi.* Nov 10 2002;82(21):1484-1487.
5. Park HS, Cheon J, Cho HY, et al. In vivo characterization of a prostate-specific antigen promoter-based suicide gene therapy for the treatment of benign prostatic hyperplasia. *Gene Ther.* Jul 2003;10(13):1129-1134.
6. Ko SC, Cheon J, Kao C, et al. Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res.* Oct 15 1996;56(20):4614-4619.
7. Denny WA. Prodrugs for Gene-Directed Enzyme-Prodrug Therapy (Suicide Gene Therapy). *J Biomed Biotechnol.* 2003;2003(1):48-70.
8. Djavan B, Nasu Y. Prostate cancer gene therapy-what have we learned and where are we going? *Rev Urol.* Fall 2001;3(4):179-186.
9. O'Keefe DS, Uchida A, Bacich DJ, et al. Prostate-specific suicide gene therapy using the prostate-specific membrane antigen promoter and enhancer. *Prostate.* Oct 1 2000;45(2):149-157.
10. Hsieh CL, Gardner TA, Miao L, Balian G, Chung LW. Cotargeting tumor and stroma in a novel chimeric tumor model involving the growth of both human prostate cancer and bone stromal cells. *Cancer Gene Ther.* Feb 2004;11(2):148-155.
11. Kubo H, Gardner TA, Wada Y, et al. Phase I dose escalation clinical trial of adenovirus vector carrying osteocalcin promoter-driven herpes simplex virus thymidine kinase in localized and metastatic hormone-refractory prostate cancer. *Hum Gene Ther.* Feb 10 2003;14(3):227-241.
12. Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for

prostate-specific antigen-positive prostate cancer cells. *Cancer Res.* Jul 1 1997;57(13):2559-2563.

13. Galanis E, Vile R, Russell SJ. Delivery systems intended for in vivo gene therapy of cancer: targeting and replication competent viral vectors. *Crit Rev Oncol Hematol.* Jun 2001;38(3):177-192.
14. Freytag SO, Rogulski KR, Paielli DL, Gilbert JD, Kim JH. A novel three-pronged approach to kill cancer cells selectively: concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther.* Jun 10 1998;9(9):1323-1333.
15. Freytag SO, Khil M, Stricker H, et al. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res.* Sep 1 2002;62(17):4968-4976.
16. Freytag SO, Stricker H, Pegg J, et al. Phase I study of replication-competent adenovirus-mediated double-suicide gene therapy in combination with conventional-dose three-dimensional conformal radiation therapy for the treatment of newly diagnosed, intermediate- to high-risk prostate cancer. *Cancer Res.* Nov 1 2003;63(21):7497-7506.
17. Lee SJ, Kim HS, Yu R, et al. Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Mol Ther.* Sep 2002;6(3):415-421.
18. Li X, Zhang YP, Kim HS, et al. Gene therapy for prostate cancer by controlling adenovirus E1a and E4 gene expression with PSES enhancer. *Cancer Res.* Mar 1 2005;65(5):1941-1951.
19. Wang JQ, Zheng QH, Fei X, Mock BH, Hutchins GD. Novel radiosynthesis of PET HSV-tk gene reporter probes [18F]FHPG and [18F]FHBG employing dual Sep-Pak SPE techniques. *Bioorg Med Chem Lett.* Nov 17 2003;13(22):3933-3938.
20. Matsubara S, Wada Y, Gardner TA, et al. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res.* Aug 15 2001;61(16):6012-6019.
21. DeWeese TL, van der Poel H, Li S, et al. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res.* Oct 15 2001;61(20):7464-7472.
22. Sadeghi H, Hitt MM. Transcriptionally targeted adenovirus vectors. *Curr Gene Ther.* Aug 2005;5(4):411-427.
23. Bettencourt MC, Bauer JJ, Sesterhenn IA, Mostofi FK, McLeod DG, Moul JW. Ki-67 expression is a prognostic marker of prostate cancer recurrence after radical

prostatectomy. *J Urol.* Sep 1996;156(3):1064-1068.

24. Horninger W, Berger AP, Rogatsch H, et al. Characteristics of prostate cancers detected at low PSA levels. *Prostate.* Feb 15 2004;58(3):232-237.

**Table 1** ***Tissue/tumor-specific replication ability of Ad-IU-1*** [a]

| Cell lines | Input Doses[b] (IFU) | Output Viral Doses [c] (LD50[d]) | |
|---|---|---|---|
| | | AdIU-1 | AdE4 |
| C4-2 | $6.6\times10^4$ | $10^6$ | $10^6$ |
| CWR22rv | $2\times10^4$ | $10^6$ | $10^6$ |
| PC-3 | $2.3\times10^5$ | $10^2$ | $10^2$ |
| DU145 | $1.6\times10^5$ | $5\times10^2$ | $5\times10^2$ |

[a] Cells were seeded and infected with AdE4PSESE1a or AdIU-1, and the supernatants were harvested for titer assay as described in "Materials and Methods".

[b] Input viral doses mean the virus doses used to infect cells.

[C] Output viral doses mean the titered virus doses in titer assay.

[d] The virus production was expressed as a LD50 value (the dilution factor that caused a CPE in at least 4 wells of cells in a row on a 96-well plate on day 7).

**Figure 1.**

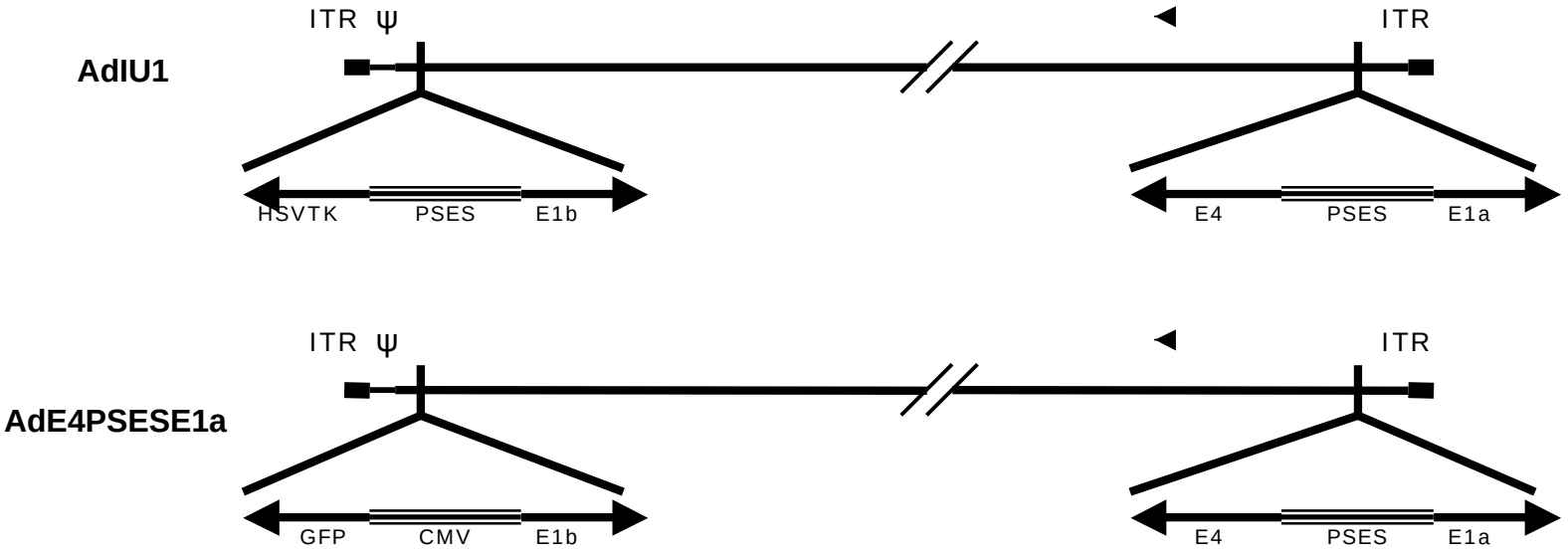

AdIU1
ITR
ψ
ITR
HSVTK
PSES
E1b
E4
PSES
E1a
AdE4PSESE1a
ITR
ψ
ITR
GFP
CMV
E1b
E4
PSES
E1a

**Figure 2.**

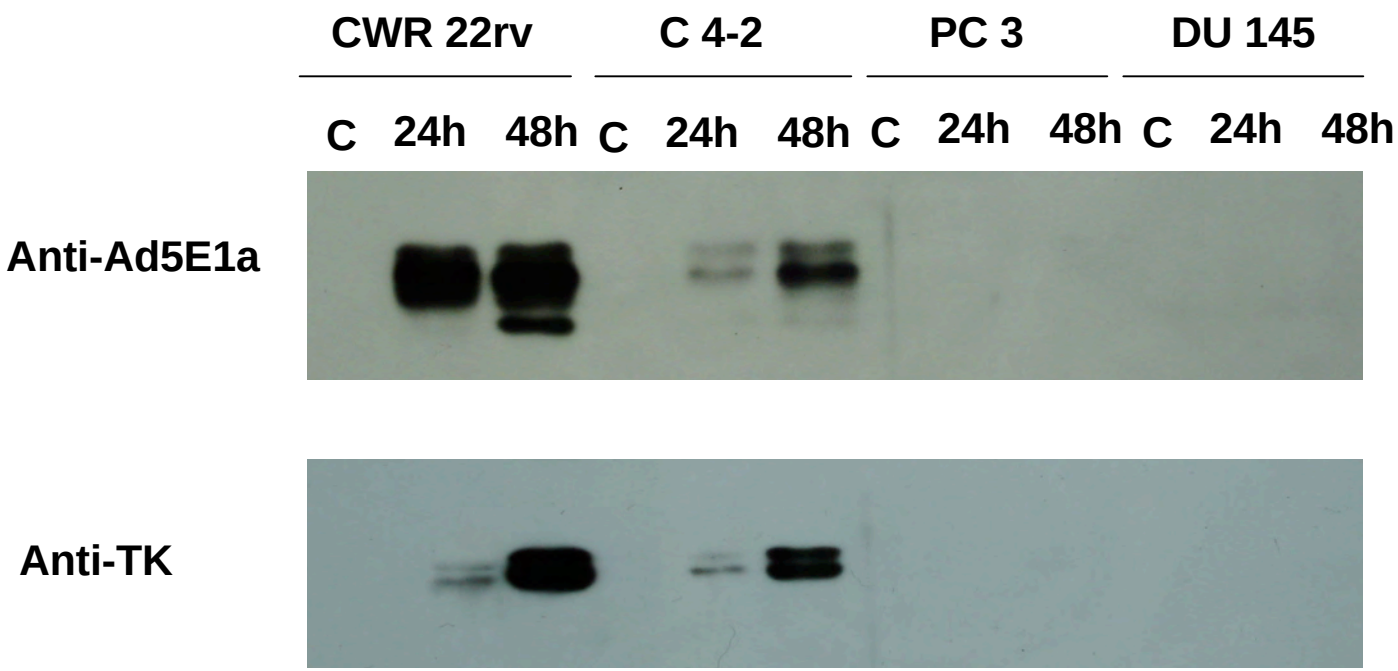

CWR 22rv
C 4-2
PC 3
DU 145
C 24h 48h C 24h 48h C 24h 48h C 24h 48h
Anti-Ad5E1a
Anti-TK

Figure 3.

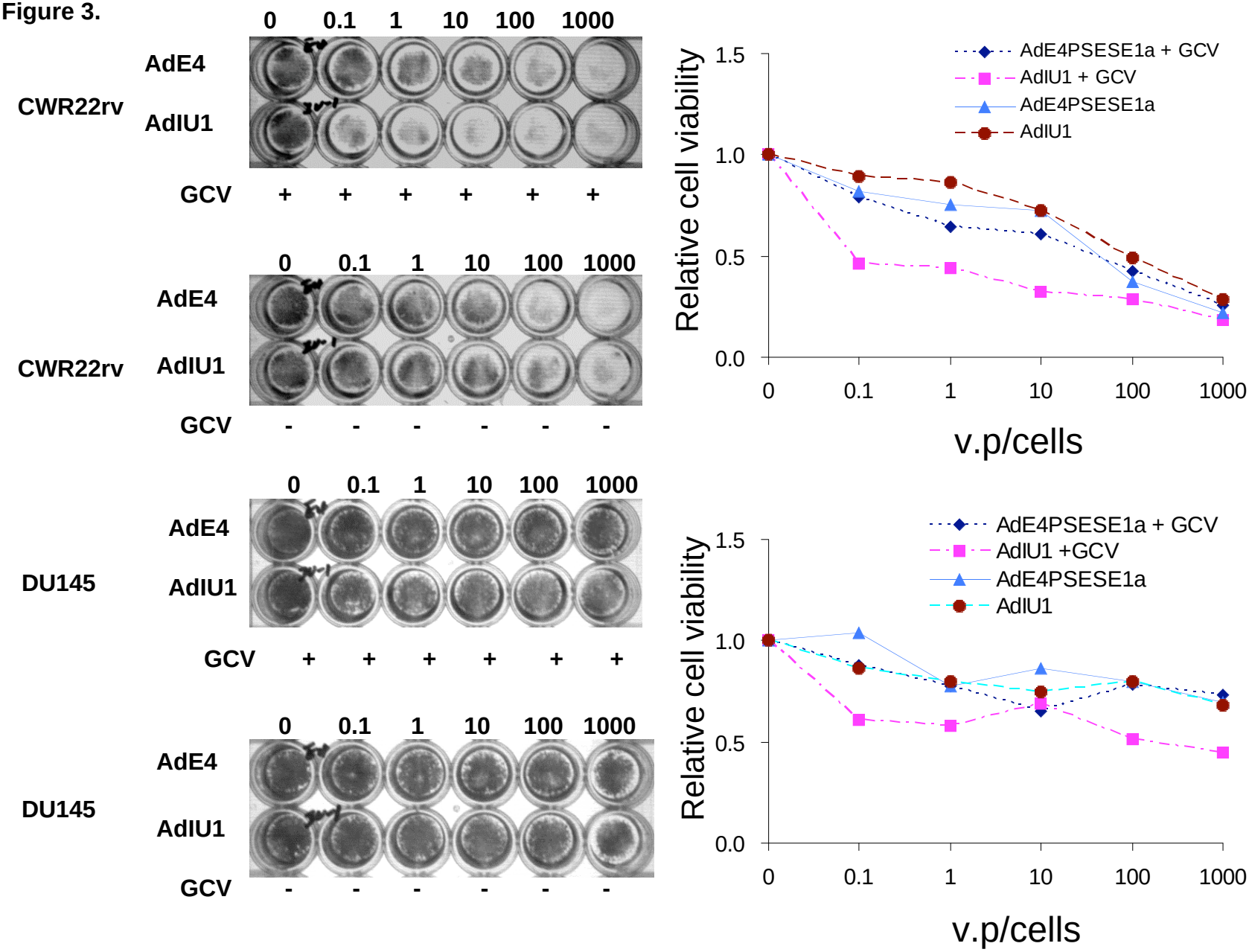

0 0.1 1 10 100 1000
AdE4
AdIU1
CWR22rv
GCV + + + + + +
0 0.1 1 10 100 1000
AdE4
AdIU1
CWR22rv
GCV - - - - - -
0 0.1 1 10 100 1000
AdE4
AdIU1
DU145
GCV + + + + + +
0 0.1 1 10 100 1000
AdE4
AdIU1
DU145
GCV - - - - - -
Relative cell viability
1.5
1.0
0.5
0.0
0 0.1 1 10 100 1000
v.p/cells
AdE4PSESE1a + GCV
AdIU1 + GCV
AdE4PSESE1a
AdIU1
Relative cell viability
1.5
1.0
0.5
0.0
0 0.1 1 10 100 1000
v.p/cells
AdE4PSESE1a + GCV
AdIU1 +GCV
AdE4PSESE1a
AdIU1

**Figure 4.**

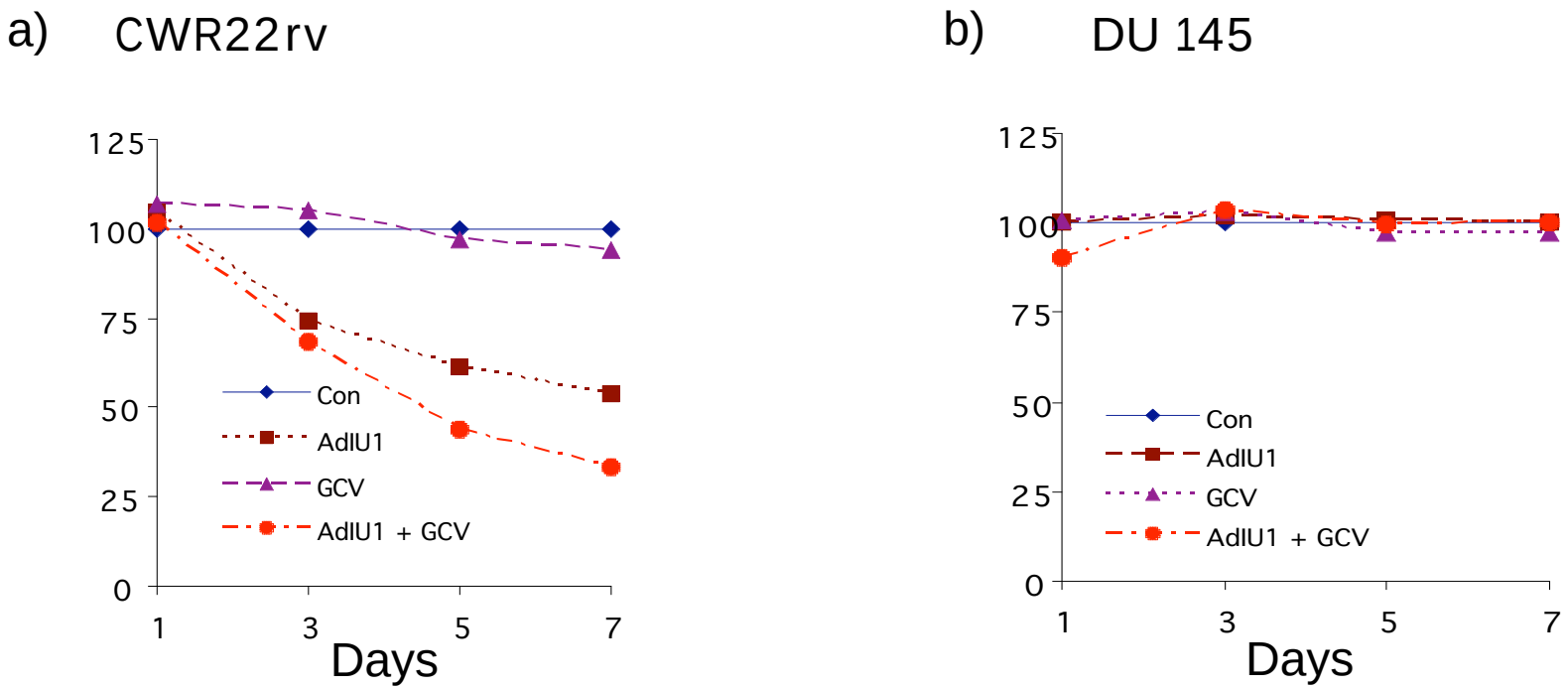
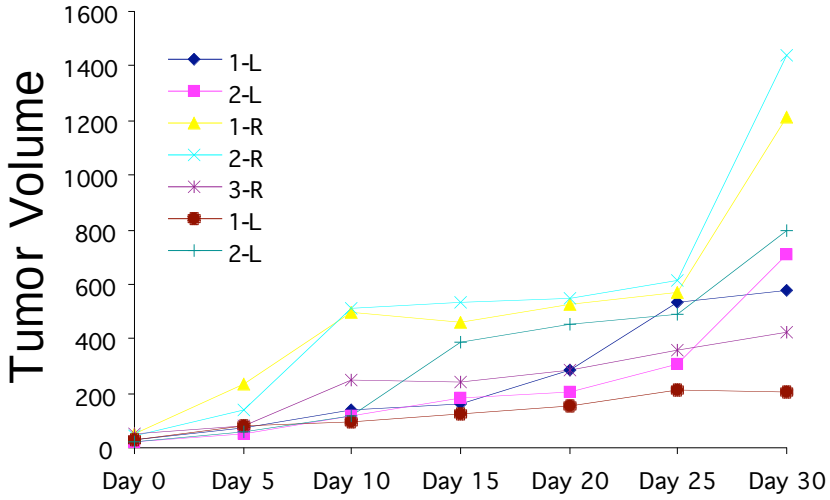
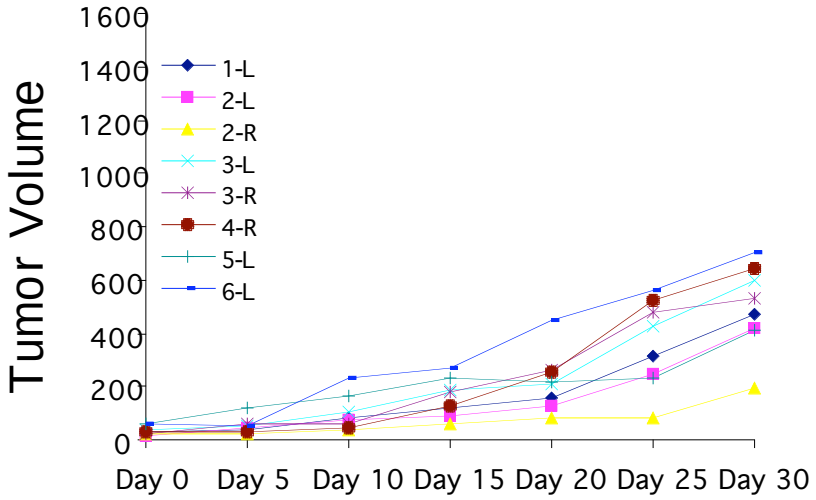

a)
CWR22rv
125
100
75
50
25
0
1
3
5
7
Days
Con
AdIU1
GCV
AdIU1 + GCV
b)
DU 145
125
100
75
50
25
0
1
3
5
7
Days
Con
AdIU1
GCV
AdIU1 + GCV

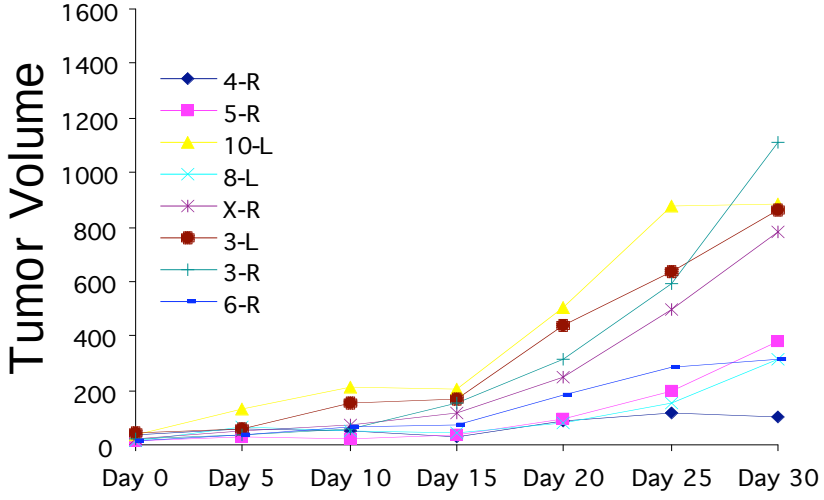
**Figure 5.**



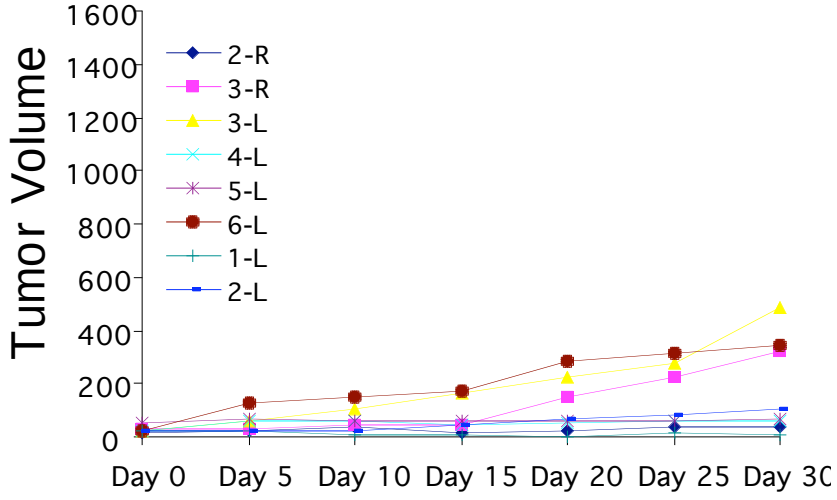

AdE4PSESE1a




AdE4PSESE1a+GCV




AdIU1




AdIU1+GCV

**Figure 5.**

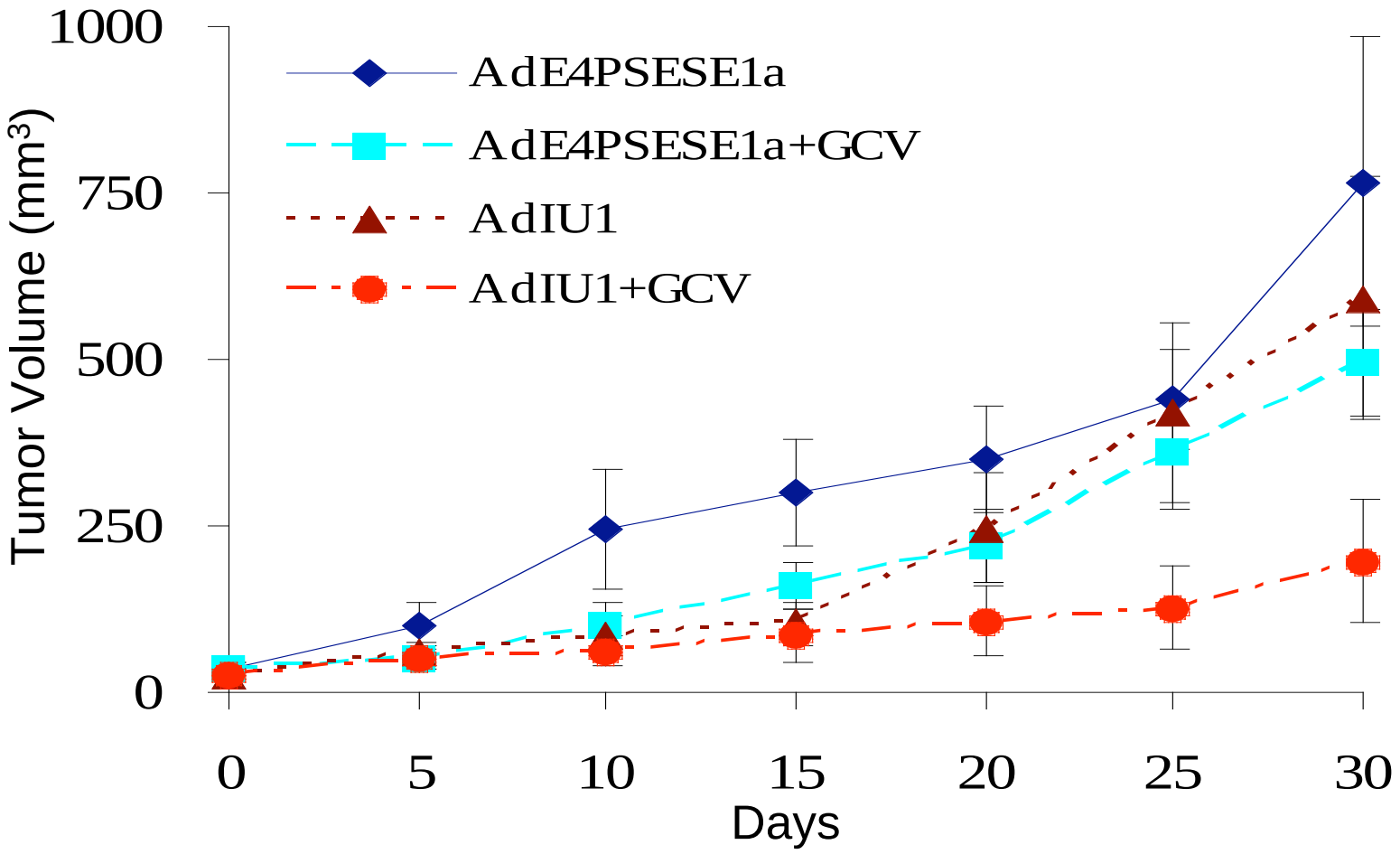

1000
750
500
250
0
Tumor Volume (mm³)
AdE4PSESE1a
AdE4PSESE1a+GCV
AdIU1
AdIU1+GCV
0
5
10
15
20
25
30
Days

**Figure 6.**